Angiotensin II in cardiac pressure-overload hypertrophy in fetal sheep

JEFFREY L. SEGAR,1 GREGORY B. DALSHAUG,2 KURT A. BEDELL,1 OLIVA M. SMITH,1 AND THOMAS D. SCHOLZ1
1Department of Pediatrics and 2Department of Surgery and the Cardiovascular Center, University of Iowa, Iowa City, Iowa 52242

Received 29 March 2001; accepted in final form 23 August 2001

Segar, Jeffrey L., Gregory B. Dalshaug, Kurt A. Bedell, Oliva M. Smith, and Thomas D. Scholz. Angiotensin II in cardiac pressure-overload hypertrophy in fetal sheep. Am J Physiol Regulatory Integrative Comp Physiol 281: R2037–R2047, 2001.—We previously demonstrated in fetal sheep that blockade of ANG II type 1 (AT1) receptors did not attenuate an increase in right ventricle (RV) mass resulting from partial occlusion of the pulmonary artery (PA). We have now determined the effects of AT2-receptor blockade (PD-123319, 10 mg·kg−1·day−1 continuous iv) on the response of the fetal RV to PA banding for 7 days. Four groups of fetuses (each n = 7) were studied beginning at 126 ± 1 days gestation (term 145 days). RV weight-to-body weight ratio (RV wt/body wt) increased (P < 0.05) in PA-banded (6.00 ± 0.27 g/kg) compared with control (5.17 ± 0.17 g/kg) and PA-banded + PD-123319 (6.19 ± 0.27 g/kg) compared with control (5.17 ± 0.17 g/kg) and PD-123319-infused (5.27 ± 0.56 g/kg) fetuses (means ± SE). Blood pressure and heart rate were similar in all groups. PD-123319 produced a decrease (P < 0.05) in AT1 but not AT2 mRNA levels in both ventricles. To examine the effect of ANG II on fetal heart growth, twin fetal sheep were infused with either ANG II (twin received vehicle) or phenylephrine (Phe) (twin received vehicle) continuously for 7 days. Mean arterial blood pressure was 20–25 mmHg higher in ANG II and Phe fetuses compared with controls. The rate-pressure product was similar in ANG II and Phe fetuses and 40–50% greater than controls. Phe resulted in no change in RV wt/body wt or left ventricle-to-body weight ratio (LV wt/body wt) compared with controls. In contrast, ANG II produced a selective increase (27 ± 5%, P < 0.05) in LV wt/body wt; no effect was seen on the RV. ANG II produced a decrease (P < 0.05) in LV but not RV AT1 mRNA levels compared with controls; no effect was seen with Phe. The data demonstrate that in the ovine fetus, AT2 receptors do not contribute to the maintenance of blood pressure or the development of pressure-overload RV hypertrophy. Elevated ANG II levels produce a selective increase in LV mass in the fetal sheep that is, in part, independent of increased systolic load.

The adult heart responds to an increased systolic pressure load by undergoing a series of adaptive structural (e.g., cardiomyocyte hypertrophy) and metabolic responses that provide more efficient myocardial performance. The morphological changes accompanying ventricular hypertrophy have been known for years, although the events initiating the complex cascade of responses are just beginning to be understood. These cellular events, which include activation of important phosphatases such as calcineurin, mitogen-activated protein kinases, and induction of protooncogenes, result in reexpression of a number of embryonic and fetal genes (for reviews, see Refs. 41 and 51).

Although an intact renin-angiotensin system (RAS) is not obligate for the development of pressure-overload hypertrophy (41), several lines of evidence suggest that ANG II plays a critical role in mediating myocardi
dial hypertrophy. ANG II has direct effects on cardiac function and structure, inducing several growth-promoting genes, protein synthesis, and cell growth (42, 46). In the mature heart, infusion of ANG II stimulates the development of cardiac hypertrophy independently of effects on blood pressure (10, 23), whereas blockade of the RAS with a converting-enzyme inhibitor or an ANG II type 1 (AT1)-receptor antagonist attenuates the development of pressure overload-induced hypertrophy and inhibits many of the molecular and cellular adaptations to pressure-overload states (33, 40).

Despite the abundant studies investigating the effects of ANG II on the mature heart, little is known regarding the role of the RAS in cardiac growth during development. In whole rat embryo culture, ANG II stimulates ventricular growth and myocyte hypertrophy, whereas blockade of AT1 and AT2 receptors inhibits ventricular development (36). Treatment of pregnant rats with an AT1- or AT2-receptor antagonist results in reduced cardiac type I collagen and transforming growth factor-β1 mRNA expression and collagen content in the newborn heart (24). During the immediate postnatal period, treatment of newborn piglets with an angiotensin-converting enzyme inhibitor or an AT1-receptor antagonist attenuates the rapid growth of the left ventricle (LV) that normally occurs in the first 3 days of life (5). Taken together, these findings suggest the RAS may be important for fetal cardiac development.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
In contrast to studies in adult animals that have shown that inhibition of the AT₁ receptor prevents the increase in ventricular mass accompanying pressure overload, we recently demonstrated that AT₂-receptor blockade failed to attenuate the development of increased right ventricle (RV) mass in pulmonary artery (PA)-banded fetal sheep (48). A possible explanation for this finding is the marked difference in expression of AT₁ and AT₂ receptors in fetal compared with postnatal myocardium (30, 43, 47). Accordingly, the goals of the present study were 1) to assess the role of AT₂ receptors in the development of pressure overload-induced cardiac hypertrophy in fetal sheep, 2) to determine if other methods of pressure overload result in increased ventricular mass, 3) to examine if ANG II exerts a growth-promoting effect on the fetal myocardium, and 4) to determine if an increased systolic pressure load alters the steady-state expression of myocardial AT₁ receptor mRNA.

METHODS

PA Banding Studies

Animals and surgical preparation. Studies were performed in fetal sheep of Dorset and Suffolk mixed breeding, obtained from a local source. The gestational ages of the fetuses were based on the induced ovulation technique (22). Fetal body weight was estimated according to the following formula: weight (kg) = 0.0961 × gestational age (days) − 9.2228; r = 0.85 (Ref. 39). All procedures were performed within the regulations of the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Iowa Animal Care and Use Committee.

Only pregnant ewes at 125–127 days gestation (term 145 days) were used for the study. Anesthesia was induced with 12 mg/kg of thiopental sodium (Pentothal Sodium, Abbott Laboratories) and maintained with a mixture of halothane (1%), oxygen (33%), and nitrous oxide (66%). Under sterile conditions, the uterus was opened over the fetal hindlimbs, and polyethylene catheters were placed into the fetal femoral arteries and veins. A catheter for measurement of amniotic pressure was secured to the fetal skin. Fetal skin incisions were closed, and the fetus was returned to the uterus. Through a separate uterine incision, the fetal head and thorax were exposed. Via a third-interspace thoracotomy, the pericardium was incised, and the main PA was exposed and isolated proximal to the ductus arteriosus. One group of fetuses had an umbilical tape ligature double-wrapped around the PA to constrict the diameter of the artery by 50%. Previous studies by us demonstrated this degree of constriction (48). A separate group of fetuses underwent a sham procedure with the umbilical tape initially wrapped around the PA and then removed. The fetal chest was closed and the hysterotomy repaired. At the completion of surgery, maternal incisions were closed in separate layers, and all catheters were exteriorized through a subcutaneous tunnel and placed in a cloth pouch on the ewe’s flank. Ampicillin sodium (Wyeth Laboratories, Philadelphia, PA) was administered intra-amniotically at the completion of surgery (2 g) and to the ewe before surgery (2 g) and daily for 3 days. Pregnant ewes were returned to individual pens and allowed free access to food and water.

Experimental protocol. The physiological studies were begun the day after surgical preparation. Fetal mean arterial blood pressure (MABP) and amniotic pressure were obtained using Statham P23 Db pressure transducers (Spectramed, Critical Care Division, Oxnard, CA) and a Gould recorder (Gould, Valley View, OH). Fetal MABP was corrected relative to concomitant amniotic pressure. Heart rate (HR) was monitored with a cardiotachometer triggered from the arterial pressure wave. Arterial blood was obtained from each fetus for determination of arterial blood gases and pH and plasma ANG II levels. After this initial fetal-monitoring period, fetuses were assigned to receive either the AT₂-receptor antagonist PD-123319 (10 mg·kg⁻¹·day⁻¹ continuous iv, 1.0 ml/h) daily for 7 days (a generous gift of Parke-Davis, Ann Arbor, MI) or an equivalent volume of vehicle (0.9% NaCl). Thus a total of four groups of fetuses were studied: 1) vehicle, 2) PA banding plus vehicle, 3) PD-123319, and 4) PA banding plus PD-123319 (n = 7 for each group). Fetal blood pressure, HR, and arterial blood samples were obtained daily in all fetuses. At the end of the 7-day infusion period, the ewe was then returned to the surgical area, and the fetuses were exteriorized under general anesthesia. The PA was visualized to document constriction, after which the fetus was euthanized with an overdose of pentobarbital sodium. The body weight was recorded and hearts were removed for determination of total weight and RV and LV free wall weights. Tissues from LV and RV free walls, obtained approximately midway between the apex and the atrioventricular groove, were snap-frozen in liquid nitrogen and stored at −80°C. Tissues were also placed in preweighed vials that were later weighed again, placed in an oven at 100°C for 20 h, and then reweighed for determination of wet-to-dry weight ratios.

ANG II and Phenyline Infusion Studies

Pregnant ewes at 125–127 days gestation (term 145 days) with twin fetal pregnancies were used for the study. Anesthesia and vascular catheterization of both twin fetuses were performed as described above. The physiological studies were begun 48 h after surgical preparation. After obtaining baseline hemodynamic values and arterial blood for determination of pH, blood gases, and plasma ANG II level, a continuous intravenous infusion of either ANG II (n = 5) at an initial dose of 40 ng·kg⁻¹·min⁻¹ or phenylephrine (Phe; n = 5) at an initial dose of 4–10 µg·kg⁻¹·min⁻¹ was started in one twin (infusion rate 1.0 ml/h; vehicle was 5% glucose in water), while the second twin received 5% glucose in water intravenously at 1.0 ml/h (n = 10, total). Fetal HR and systolic, diastolic, and mean blood pressures were obtained for 3–4 h after initiating infusions on the first day and for 30 min twice daily thereafter for a total of 7 days. Infusion concentrations of ANG II (range 40–60 ng·kg⁻¹·min⁻¹) and Phe (range 10–40 µg·kg⁻¹·min⁻¹) were adjusted twice daily based on blood pressure measurements with a goal of increasing MABP 20 mmHg above that of the twin control. The twice daily measurements of systolic blood pressure and HR were averaged, and the values were used to calculate the rate-pressure product (RPP; systolic blood pressure × HR), an indirect measure of myocardial work load.

Analytic procedures. Arterial blood for pH, PCO₂, and PO₂ was collected anaerobically in a heparinized syringe, and measurements were immediately determined at 39.5°C using a BGM 1302 pH/blood gas analyzer (Instrumentation Laboratory, Lexington, MA). Blood for measurement of ANG II levels was collected into prechilled EDTA tubes and centrifuged, and the plasma was frozen at −80°C. Measurements of plasma ANG II were determined by radioimmunoassay in
the University of Wisconsin School of Veterinary Medicine Radioimmunoassay Laboratory (Dr. M. Brownfield, Director) using ANG II primary (rabbit) and secondary (goat) antibodies produced by the laboratory. Intra-assay and interassay variability are 10 and 15%, respectively.

Northern blot analyses. Total cellular RNA was prepared from LV or RV free walls using a modification of the RNeasy kit (Qiagen, Valencia, CA) as previously described (45), quantitated spectrophotometrically, and stored at −80°C in ethanol until use.

Plasmids containing the ovine-specific partial cDNA clones for AT1 and AT2 receptors were linearized and used to generate a labeled antisense probe using T7 RNA polymerase as previously described (37, 38). Ovine vascular endothelial growth factor (VEGF) cDNA (kindly provided by E. Perkett, Vanderbilt University) was similarly used as a template to generate a riboprobe for VEGF mRNA. The ovine cDNA fragment included exons to the common vascular endothelial growth factor (VEGF) cDNA (kindly provided by E. Perkett, Vanderbilt University) was similarly used as a template to generate a riboprobe for VEGF mRNA.

Northern blot hybridization was performed using techniques previously described (37, 38, 43). Hybridization signals were detected and quantitated using an AMBIS 4000 Radioanalytic Imaging System (AMBIS, San Diego, CA) or using a PhosphorImager (Molecular Dynamics). Background counts above each lane were determined and subtracted from the total counts generated in each region of interest to yield a net count value. Blots quantitated by the AMBIS were also exposed to Kodak XAR film at −70°C. All blots were stripped and rehybridized with a 32P-labeled probe to the 28S unit of rRNA. Signals from the 28S-probed blots were used to correct for variable RNA loading.

Histological evaluation. Samples of LV and RV free walls (5 mm × 5 mm) were fixed with buffered 10% formalin. After several days, tissue was embedded in paraffin, cut, and stained with hematoxylin-eosin or Masson trichrome.

Data analysis. For quantitation of mRNA abundance, samples were analyzed together on a single Northern blot hybridization to control for day-to-day variations in hybridization efficiency. Northern blots were done in triplicate. Expression of AT1, AT2, and VEGF mRNA was normalized by corresponding 28S rRNA net counts.

Comparisons among the different groups were performed using two-way ANOVA, factoring for PA banding and treatment with PD-123319 (PA banding studies); repeated-measures one-way ANOVA (hemodynamic and hormonal values for infusion studies), or one-way ANOVA (tissue weights for infusion studies). When the ANOVA indicated a significant difference among groups, as indicated by the F statistic, comparison among means was performed by the Duncan multiple comparison procedure (13). Statistical significance was defined as P < 0.05, and all data are expressed as means ± SE.

RESULTS

PA Banding Studies

Effects of PA banding and PD-123319 on fetal hemodynamics and arterial blood values. The effects of PA banding, treatment with PD-123319, and concomitant PA banding and PD-123319 on fetal HR and MABP are shown in Fig. 1. Fetal HR and MABP were similar in all four groups on the first postoperative day (day 1) and remained unchanged over 7 days (day 8). Specifically, treatment with PD-123319 had no demonstrable effect on HR or MABP in either PA-banded or non-banded fetuses. Arterial blood pH, PCO2, Po2, and circulating levels of ANG II were similar among and within all groups on days 1 and 8 (Table 1).

Effects of PA banding and AT2-receptor blockade on fetal somatic and cardiac mass. No difference in total body weight was found among control (3.12 ± 0.15 kg), PA-banded (2.99 ± 0.21 kg), PD-123319-infused (3.03 ± 0.13 kg), and PA-banded + PD-123319-infused (2.95 ± 0.18 kg) fetuses. There was a significant increase in heart weight-to-body weight ratio in PA-banded (6.00 ± 0.09 g/kg) and PA-banded + PD-123319-infused (6.19 ± 0.27 g/kg) compared with control (5.17 ± 0.17 g/kg) and PD-123319-infused (5.27 ± 0.35 g/kg) fetuses (Fig. 2). This increase in heart mass appeared specific for the RV, as demonstrated by the significant increase in RV weight-to-body weight ratio in PA-banded (6.00 ± 0.09 g/kg) and PA-banded + PD-123319-infused fetuses with no change in the LV weight-to-body weight ratio. Administration of the AT2-receptor antagonist PD-123319 over the 7-day period did not alter the development of increased RV mass in PA-banded fetuses (Fig. 2). In addition, infusion of PD-123319 for 1 wk had no effect on cardiac mass, as total body weight, heart weight, and LV weight-to-body weight and RV weight-to-body weight ratios were similar between these animals and controls.
AT2 mRNA levels in either PA-banded or nonbanded fetuses. Infusion of PD-123319 also had no effect on RV or LV pressure overload resulting from PA banding was not associated with any significant alterations in expression of AT1 or AT2 mRNA in either the RV or LV. Infusion of PD-123319 also had no effect on RV or LV AT2 mRNA levels in either PA-banded or nonbanded fetuses. On the other hand, PD-123319 produced a significant decrease in AT1 mRNA levels in both ventricles of banded as well as nonbanded animals.

**Chronic Infusion Studies**

Effect of ANG II and Phe infusion on fetal hemodynamics and arterial blood values. Fetal HR and blood pressure were similar among all groups on day 1, before beginning the infusions. Average daily fetal HR (data not shown) and blood pressure (Fig. 4) were similar in the two control groups and did not change during the course of the study. Mean blood pressure significantly increased after initiation of ANG II or Phe infusion and remained elevated for all 7 days of infusion. MABP was greater in the Phe group than in the ANG II group on days 2 and 3 but was similar thereafter. HRs did not differ between the ANG II and Phe group at any time point (data not shown).

The RPP, calculated as the product of the systolic blood pressure and the HR, was used as an indirect measure of myocardial workload. The RPP was similar in the two control groups and did not change over the course of the study (Fig. 4). The RPP was significantly higher in the Phe group compared with both control groups on days 2–8 and higher than the ANG II group on days 2 and 3. The ANG II group had a RPP similar to the control groups on days 1 and 2 but was significantly greater thereafter and similar to the Phe group for days 4–8.

Arterial blood pH, PO2, and PCO2 values were similar among and within all groups throughout the experiment (data not shown) and within the normal range for our laboratory (pH 7.34–7.39, PCO2 47–52 mmHg, PO2 19–24 mmHg). As expected, circulating ANG II values were significantly greater in the ANG II-infused group than in the Phe or control groups (Fig. 5). No difference in ANG II levels was seen between the Phe group and control groups.

**Effect of ANG II and Phe infusion on fetal and organ weights.** Fetal weight at death was similar in all groups (Table 2). Infusion of ANG II led to a 27 ± 5% increase (P < 0.05) in LV free wall mass (expressed per kg fetal weight) compared with the Phe and control groups (Fig. 6). Despite a similar if not slightly greater RPP compared with the ANG II fetuses, the group infused with Phe showed no increase in LV free wall mass. No effect of ANG II or Phe infusion was seen on RV free wall weight. The LV-to-RV weight ratio (g/g) was also significantly greater in the ANG II animals compared with the other groups (Fig. 6). Phe infusion had no effect on this ratio.

Infusion of ANG II or Phe had no effect on adrenal, brain, kidney, or lung weights when expressed as organ weight per fetal weight (g/kg) (Table 2). Because prolonged infusion of angiotensin into fetal lambs promotes transplacental transfer of water from mother to fetus (2), we also assessed tissue water content in various organs. Tissue specific dry-to-wet weight ratios were similar in all groups of animals, suggesting no confounding effect of tissue water content on organ weights, including the LV (Table 3).

**Effect of ANG II and Phe infusion on steady-state, AT1, and VEGF mRNA levels.** Northern blot analysis was used to determine the effects of infusion of PD-123319 on expression of fetal cardiac AT1 and AT2 receptor mRNA. Chronic infusion studies were performed for selected genes. Infusion of PD-123319 on expression of fetal cardiac AT1 and AT2 receptor mRNA.
ANG II decreased LV AT1 mRNA levels relative to control values (Fig. 7). This change was specific for the LV, as no differences in the level of expression were seen in the RV. No effect on ventricular AT1 gene expression was seen in the Phe-infused animals (Fig. 8). Because an acute increase in ventricular mass is associated with a need for concomitant vascular growth, and ANG II may upregulate expression of VEGF (8, 54), we also determined VEGF mRNA levels in the fetal hearts. In the myocardial tissue examined, VEGF mRNA was expressed as a single band at ~3.9 kb, which would be consistent with VEGF165 mRNA. Surprisingly, Phe infusion significantly decreased VEGF mRNA levels in both LV and RV, whereas ANG II had no effect on ventricular expression of the gene (Figs. 7 and 8).

**DISCUSSION**

These experiments demonstrate that ANG II infusion induces a selective increase in LV but not RV mass in the fetal sheep heart. The absence of effect of Phe on ventricular mass suggests that ANG II regulation of LV growth in the fetus is independent of increases in systemic pressure load or cardiac work. Blockade of AT2 receptors failed to attenuate or promote the development of an increase in RV mass in PA-banded fetuses or alter cardiac mass in nonbanded fetuses. These findings complement our previous studies in which blockade of AT1 receptors with losartan had no effect on the development of RV hypertrophy produced by partial occlusion of the PA (48). Taken together, these findings suggest that activation of ANG II receptors by endogenous ANG II is not obligatory for the development of pressure overload-induced RV hypertrophy in the fetal lamb. However, ANG II appears to have specific effects in the fetal LV, although its role in promoting LV growth remains to be further defined.

The developmental expression pattern and function of AT1 and AT2 receptors are quite distinct. AT2-receptor expression is high in developing embryos and fetal mesenchymal tissues and decreases late in development and with postnatal maturation (16, 17). On the other hand, AT1 receptors appear later in fetal development and are expressed in numerous adult tissues, including adrenal, kidney, heart, liver, lung, aorta, brain, and vascular smooth muscle (for review, see Ref.
The AT1 receptor mediates numerous physiological actions of ANG II, including cardiovascular control, salt and water balance, and cell growth (9). The functions of the AT2 receptor have not been clearly defined, although recent studies suggest these receptors exert hypotensive and antiproliferative effects and inhibit the activity of growth factor signaling pathways (16).

Numerous investigations have demonstrated that in the postnatal heart, ANG II acts at the AT1 receptor to induce a fetal phenotype of gene expression and stimulate myocyte hypertrophy, fibroblast proliferation, and accumulation of the extracellular matrix (34, 42). The role of AT2 receptors in regulating growth in the heart is unclear. In vitro, treatment with an AT2-receptor antagonist has no significant effect on ANG II induction of hypertrophy or expression of protooncogenes and growth factor genes in myocytes or fibroblasts (42). Bartunek et al. (4) reported that inhibition of AT2 receptors results in amplification of the early signals of LV growth response to ANG II, consistent with the antiproliferative effects of AT2-receptor activation described in other tissues (1). On the other hand, Poole et al. (35) demonstrated that AT2 receptors contribute to the development of cardiac hypertrophy in rats with aortocaval fistulas. Targeted gene overexpression and deletion have also been used to explore the role of the AT2 receptor in the heart. The AT2 receptor-null mouse shows no gross morphological cardiac defects despite its normally extensive expression in developing heart (21). Masaki and colleagues (28) reported that cardiac-specific overexpression on the AT2 gene resulted in no obvious phenotypic or morphological changes in the myocardium, such as myocyte necrosis or fibrosis. However, because the construct contained the mouse α-myosin heavy chain (MHC) promoter and would be expected to have an expression pattern similar to that of endogenous α-MHC, the AT2 transgene would not be expressed during fetal development.

A major difference in studying the effect of ANG II-receptor blockade on pressure overload-induced hypertrophy in fetal compared with postnatal animals is that in the fetal heart, AT2 receptors are abundantly expressed. We previously demonstrated in the ovine heart that AT1 mRNA levels remain relatively constant in all four cardiac chambers between 95 days gestation (term 145 days) and 8 wk of age (43). In contrast, cardiac AT2 mRNA levels rapidly decrease during the first week of postnatal life. Studies in a number of other species have shown similar developmental regulation of cardiac ANG II receptors (47). We therefore took advantage of this unique model to investigate a potential role for AT2 receptors in the development of load-induced increase in fetal ventricular mass. Blockade of the AT2 receptor for 7 days had no demonstrable effect on the increase in RV mass in PA-banded fetuses or on normal cardiac or somatic mass in non-PA-banded fetuses. Although this finding is not surprising, it is possible that any effect was too small to be detected by our study. In addition, we did not undertake any detailed morphological or histochemical examination of the tissue to determine if differences in myocyte morphology or extracellular matrix or evidence for tissue remodeling/apoptosis were present. In the fetal sheep heart, increased RV load from PA banding results in both hyperplastic and hypertrophic myocyte growth (3). It is possible that while having no effect on change in RV mass in response to PA banding, selective AT2 (present study)- or AT1 (previous study)-receptor blockade may differentially regulate the hyperplastic and hypertrophic processes of the fetal heart.
A limiting factor in this study is the inability to determine the extent to which AT2-receptor blockade was achieved. In preliminary studies, we attempted to determine the optimal dose by taking advantage of the purported hypotensive effect of AT2-receptor stimulation (18, 21). We hypothesized that in fetuses subjected to peripheral AT1-receptor blockade, concomitant blockade of AT2 receptors would result in an increase in blood pressure because the effect of endogenous ANG II would be removed. In a similar fashion, we speculated that after AT1-receptor blockade, infusion of ANG II would result in slight hypotension, due to selective activation of AT2 receptors, and that this response could be blocked by administration of the AT2-receptor antagonist. Unfortunately, neither proved to be the case. Therefore, the dose chosen was similar to that reported for in vivo studies in postnatal animals (27). We did observe a molecular response to infusion of PD-123319, i.e., a significant downregulation of AT1-receptor protein levels are similar in the LV and RV at this stage of fetal development (43, 48) and mass failed to increase during infusion of ANG II. The reason for this ventricle-dependent response is unclear. In contrast, although the genes reexpressed during the development of hypertrophy in the adult are already activated and the genes reexpressed during the development of hypertrophy in the adult are already activated and 2) there exists a different balance of AT1- and AT2-receptor expression compared with the postnatal heart. Our findings demonstrate that increased circulating levels of ANG II selectively increase LV but not RV mass in the fetal sheep heart. This response appears to be independent of ANG II-mediated changes in blood pressure for several reasons. First, neither ventricle in Phe-infused fetuses showed an increase in mass despite experiencing increases in MABP and RPP similar to those of the ANG II-infused fetuses. Second, the unrestricted patent ductus arteriosus allows the RV to experience similar systolic loads, yet RV mass failed to increase during infusion of ANG II. Reasons for this ventricle-dependent response are unclear. Ovine cardiac AT1 and AT2 gene expression and AT1-receptor protein levels are similar in the LV and RV at this stage of fetal development (43, 48) and therefore unlikely to contribute to this ventricle-dependent response.

This finding is somewhat surprising given that AT1-receptor expression is increased in the vascular smooth muscle of AT2-deficient mice (52) and that various authors have observed negative cross-talk between AT1 and AT2 receptors at both the functional and intracellular signaling level (16, 19, 29). We did not determine whether AT1 gene expression was altered on myocytes, nonmyocytes, or both cell types or if similar effects of AT2 blockade on AT1 mRNA levels in the myocardium are present on other fetal tissues. Additional studies will be needed to determine if the effects of AT2-receptor blockade on cardiac AT1 receptor mRNA levels are mediated through direct signaling pathways or indirectly via altered cardiovascular or neurohumoral function.

Infusion of pressor and subpressor doses of ANG II in mice and rats increases LV mass and induces expression of fetal-type cardiac genes, including atrial natriuretic peptide, ß-actin, and ß-MHC (23, 50). Administration of hydralazine to normalize blood pressure changes does not block the development of cardiac hypertrophy or alter changes in selective cardiac gene expression, suggesting the remodeling and reprogramming of gene expression are independent of elevation in blood pressure. Before this study, there was little information regarding whether ANG II can stimulate cardiac growth in the fetus, at a time when 1) many of the genes reexpressed during the development of hypertrophy in the adult are already activated and 2) there exists a different balance of AT1- and AT2-receptor expression compared with the postnatal heart. Our findings demonstrate that increased circulating levels of ANG II selectively increase LV but not RV mass in the fetal sheep heart. This response appears to be independent of ANG II-mediated changes in blood pressure for several reasons. First, neither ventricle in Phe-infused fetuses showed an increase in mass despite experiencing increases in MABP and RPP similar to those of the ANG II-infused fetuses. Second, the unrestricted patent ductus arteriosus allows the RV and LV to experience similar systolic loads, yet RV mass failed to increase during infusion of ANG II. Reasons for this ventricle-dependent response are unclear. Ovine cardiac AT1 and AT2 gene expression and AT1-receptor protein levels are similar in the LV and RV at this stage of fetal development (43, 48) and therefore unlikely to contribute to this ventricle-dependent response.
We originally hypothesized that the increased systolic load produced by ANG II and Phe would induce greater changes in the RV than in the LV. Because of differences in fetal LV and RV dimensions and the curvature of the free wall, RV systolic wall stress is greater than that of the LV (in the presence of similar PA and aortic pressures, which is the usual case in the fetal circulation) (32). When afterload is increased, the fetal ventricles are affected in a quantitatively different manner; fetal RV wall stress increases by a greater amount than that of the LV. The RV of the fetus is also exquisitely sensitive to afterload, and increased systolic load may affect end-diastolic volume and stroke volume to a far greater extent in the RV than the LV. It is possible that the mechanical stretch imposed on the fetal ventricle by partial occlusion of the PA or aorta induces different signaling pathways than do pharmacologically mediated increases in afterload. Furthermore, a biventricular increase in load, as produced in this study, may have different effects on the heart than a univentricular load. If the increases in afterload are balanced between the ventricles, the hemodynamic and mechanical effects may be minimized.

The failure of Phe infusion, which greatly increased fetal blood pressure and led to a near doubling of the cardiac RPP, to affect either LV or RV mass was surprising. The fetal heart is clearly capable of increasing in mass in response to increased load, as demonstrated by the increase in RV and LV mass accompanying constrictive banding of the fetal proximal PA or aorta, respectively (6). Furthermore, α1-adrenergic stimulation induces a hypertrophic response in cultured neonatal and adult cardiac myocytes, as well as in the postnatal heart, in vivo (44, 49, 53). Further study of the signaling mechanisms and function of fetal cardiac α-adrenoreceptors is needed to understand this finding.

In fetal sheep, ANG II has been shown to promote transplacental transfer of water to the fetus and accumulation of fetal fluids (2). The exact mechanism(s) by which ANG II increases water supply to the fetus are not known but may be related to changes in membrane permeability or filtration coefficients to salts or altered cotyledon capillary hydrostatic pressure (12). Although we did not measure intra- and extracellular fluid volumes, no fetus appeared edematous. Grossly, no polyhydramnios was present, although amniotic and allantoic fluid volumes were not measured. Importantly,

**Table 3. Tissue water content in fetuses infused with ANG II or Phe**

<table>
<thead>
<tr>
<th></th>
<th>Left Ventricle</th>
<th>Right Ventricle</th>
<th>Skeletal Muscle</th>
<th>Brain</th>
<th>Kidney</th>
<th>Liver</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANG II infused</td>
<td>0.173 ± 0.005</td>
<td>0.180 ± 0.006</td>
<td>0.181 ± 0.002</td>
<td>0.142 ± 0.002</td>
<td>0.149 ± 0.009</td>
<td>0.223 ± 0.007</td>
<td>0.106 ± 0.014</td>
</tr>
<tr>
<td>Control (ANG II)</td>
<td>0.170 ± 0.008</td>
<td>0.180 ± 0.006</td>
<td>0.150 ± 0.002</td>
<td>0.130 ± 0.007</td>
<td>0.179 ± 0.005</td>
<td>0.220 ± 0.012</td>
<td>0.104 ± 0.004</td>
</tr>
<tr>
<td>Phe infused</td>
<td>0.192 ± 0.011</td>
<td>0.198 ± 0.006</td>
<td>0.151 ± 0.006</td>
<td>0.149 ± 0.005</td>
<td>0.171 ± 0.003</td>
<td>0.241 ± 0.005</td>
<td>0.107 ± 0.013</td>
</tr>
<tr>
<td>Control (Phe)</td>
<td>0.177 ± 0.008</td>
<td>0.202 ± 0.015</td>
<td>0.132 ± 0.004</td>
<td>0.131 ± 0.002</td>
<td>0.189 ± 0.006</td>
<td>0.214 ± 0.008</td>
<td>0.102 ± 0.004</td>
</tr>
</tbody>
</table>

Values are means ± SE of dry-to-wet tissue weight ratios; n = 5 in each group. Control (ANG II) represents twin of fetus that received ANG II infusion; control (Phe) represents twin of fetus that received Phe infusion. Control animals received infusion of vehicle, 5% dextrose in water, at 1 ml/h.
tissue-specific dry-to-wet ratios were similar in all three groups, suggesting there was no confounding effect of tissue water content on the measured heart weights.

Prolonged infusion of ANG II resulted in decreased expression of AT1 mRNA in the LV but not the RV. It is unlikely this response is directly related to an increase in systolic pressure load because no change in the expression of AT1 mRNA was seen in the LV of Phe-infused fetuses. In cultured myocytes and cardiac fibroblasts, ANG II results in a concentration-dependent decrease in AT1 mRNA expression (11). Our current results are in contrast to our previous findings, in which infusion of ANG II for 24 h, which increased MABP by ~22 mmHg, had no effect on steady-state AT1 mRNA levels. Differences in the duration of the infusion and the imposed hemodynamic alterations as well as the secondary effects of elevated blood pressure on other circulating hormones and potential regulators of AT1 gene expression may contribute to these discrepant results.

ANG II has been demonstrated to stimulate angiogenesis in a number of tissue types (20, 25) that may, in part, be mediated by VEGF. VEGF mRNA exists as five transcripts that are derived by alternative splicing of a single precursor mRNA. The smaller isoforms, VEGF121 and VEGF165, are soluble and are secreted as homodimeric glycoproteins, whereas the larger isoforms, VEGF189 and VEGF206, are almost completely bound in the extracellular matrix and may serve as storage forms of VEGF. VEGF165 is the most abundant form and in vitro studies the most biologically active form (15). ANG II acts via AT1 to upregulate mRNA levels of VEGF and the VEGF receptor VEGFR1 (8, 31). Furthermore, myocardial stretch or pressure load induces VEGF expression in the heart (26). We therefore determined the effects of ANG II and Phe infusion on steady-state VEGF mRNA in fetal ventricle. Prolonged infusion of Phe but not ANG II resulted in downregulation of VEGF gene expression in both ventricles. The lack of increase in VEGF expression after 7 days of increased systolic load is not surprising given that others have shown that, in response to acute pressure overload, there is an increase in VEGF mRNA levels within 3–6 h that returns to control levels by 24 h (7). The relative decrease in VEGF mRNA seen after 7 days of Phe compared with controls may be related to myocardial remodeling and changes in vasculature that take place during this time period. It is unlikely that a direct effect of selective activation of cardiac α-adrenoreceptors by Phe is responsible, as previous studies have shown that selective activation of α1- or α2-adrenoreceptor stimulation has no effect on VEGF gene expression (14). On the other hand, indirect effects via elevation in blood pressure on gene expression may occur. Nonetheless, it appears that in the face of pharmacologically increased afterload, VEGF mRNA expression in the fetal heart is maintained by ANG II relative to Phe.

Perspectives

In the adult heart, ANG II plays an important role in cardiac performance, remodeling, and genetic reprogramming in response to cardiac disease and pathological states. The perinatal heart provides a novel model to explore ANG II-mediated mechanisms that regulate a variety of physiological processes that are important not only for postnatal heart growth but also for the adaptation to cardiac disease.

The functional and morphological differences between the fetal RV and LV have been appreciated for some time. The present study provides new information regarding the responses of the fetal ventricles to increased systolic load and the differential responses of the ventricles after stimulation by ANG II. Continued investigation of the effects of ANG II on the fetal and early postnatal heart is essential if we are to better understand the important role of ANG II in modulating cardiac function at all stages of development.

We thank M. A. Hart for assistance in preparing this manuscript.

REFERENCES


